

## Labeling Pattern of Translocated Stachyose in Squash<sup>1</sup>

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**Abstract.** One mature blade of each squash plant was continuously labeled with  $^{14}\text{CO}_2$  for 15, 30, or 70 minutes in light. The ethanol soluble materials from serial sections of petioles were extracted and separated by paper chromatography. The ratios of label in the various components of this fraction were determined. Stachyose, which contained the major portion of the label of this fraction, was hydrolyzed and the resultant hydrolysate was separated by paper chromatography. Specific activities of the hexoses derived from stachyose were determined. It was found that the glucose and fructose moieties of stachyose were labeled at the same rates; however, the galactose moiety became labeled more rapidly.

Sucrose has been implicated as the major molecule of phloem transport in most species studied [see reviews by Kursanov (8), Swanson (15), and Zimmermann (25)]. Stachyose, along with other members of the raffinose family of oligosaccharides, only recently has been implicated as an important transport molecule in certain woody species, notably *Fraxinus americana* L. (18, 22, 23, 24); in *Verbascum thapsus* L. and in various cucurbits (13, 19, 20, 21, 22).

Direct evidence has been published establishing that translocated sucrose becomes equally labeled in the 2 hexose moieties when  $^{14}\text{C}$  labeled compounds are supplied to the leaves (6). The same inference can reasonably be drawn from the data presented by Swanson and El Shishiny (16) and by Burley (2). Recently data have been published which indicate that photosynthetically produced stachyose becomes labeled more rapidly in the galactose moiety than in the other 2 hexose moieties (7, 14); however, that work was not extended to the study of the labeling sequence of translocated stachyose. This report is devoted primarily to the time course labeling pattern of the hexose moieties of translocated stachyose. Since stachyose has been established as a transport molecule in cucurbits (13, 19, 20, 21), straight-necked squash was selected.

## Materials and Methods

**Plant Material.** Seeds of *Cucurbita pepo* L., var. melopepo torticollis Bailey, were germinated in a moist chamber at  $28 \pm 1^\circ$  for 4 days, maintained in vermiculite for an additional 4 days, then the seedlings were transferred to modified Meyer's solution<sup>3</sup> (9) in a Sherer-Gillett Chamber, Model 512-37. The light intensity supplied with a combination of fluorescent and incandescent lamps was 1400 ft-c, the temperature was  $24 \pm 1^\circ$  during the 17 hour light period and  $18 \pm 1^\circ$  during the dark period. After 7 days in this environment, the plants were transferred to conditions of continuous temperature of  $26 \pm 3^\circ$  and 1000 ft-c supplied with 300-w reflector flood incandescent lamps. The day before each experiment, 22 or 23 days from seed, all leaves except those at nodes 4 and 7 were removed.

**Experimental Procedure.** The apparatus used for supplying a continuous, constant supply of  $^{14}\text{CO}_2$  was illustrated by Geiger and Swanson (5). Five hours before labeling started, the mature (source) blade at node 4 was sealed into the plastic cuvette, the lights were turned on, and laboratory air was directed through the cuvette.

After labeling periods of 15, 30, or 70 minutes, the petiole was excised, cut into 2 cm sections which were placed in individual vials containing hot 95% ethanol. The samples were stored at about  $-20^\circ$ ; these same storage conditions were provided during interruptions in the subsequent analytical steps.

**Extraction and Purification of Labeled Sugars.** Petiole sections were extracted for 24 hours in a micro-soxhlet extraction bank with 80% (v/v) ethanol. The extracts were dried at about  $35^\circ$  using reduced pressure, then dissolved in 40% (v/v) ethanol and streaked on Whatman No. 1 filter paper for chromatography.

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<sup>3</sup>  $\text{FeCl}_3$  was replaced with Sequestrene-Na-Fe supplying 5 mg/l, also 1 mM NaCl was added.

The initial identification of the labeled stachyose was based on co-chromatography with known stachyose in 2 *n*-butanol-acetic acid-water (BAAW) systems, 3:3:2 v/v/v and 1:1:1 v/v/v; *n*-butanol-ethanol-water (BEW) 9:1:10 v/v/v, organic phase; phenol-water 4:1 w/v; and isopropanol-pyridine-water-acetic acid 8:8:4:1 v/v/v/v. The 1:1:1 BAAW system was used for the purification of all stachyose from which data were collected. An autoradiogram of such a chromatogram is illustrated in figure 1. The  $R_F$  values in this system, taking sucrose as 1.00 were: stachyose, 0.54; raffinose, 0.76; galactose, 1.05, glucose, 1.08; fructose, 1.18.

*Determination of the Distribution of  $^{14}C$ .* The developed chromatograms were scanned with a specially built apparatus (22). If the scan peaks for the oligosaccharides overlapped, the oligosaccharides were re-chromatographed. The relative  $^{14}C$  content (tables I and II) were obtained by

Table I. *Distribution of  $^{14}C$  In Petioles*

The proportional detectable  $^{14}C$  extracted from 2 cm serial sections of petiole. Maximum activity within each petiole is taken as 100. Section 1 was proximal to the blade.

Sec. no.	Relative total activity among sections (max. = 100)		
	70 Min labeling Expt. 1	30 Min labeling Expt. 2	15 Min labeling Expt. 3
1	82	84	100
2	59	100	83
3	58	96	72
4	57	83	77
5	58	86	50
6	69	62	44
7	63	83	37
8	64	76	30
9	60	76	18
10	60	69	15
11	82	73	8
12	86	53	0
13	89	60	0
14	91	59	0
15	67	45	0
16	84	55	0
17	100	...	...

Table II. *The Proportional Activity in Designated Compounds From Petiolar Extract*

Labeling time	Expt no.	Radioactivity in each compound				
		VERB	STACH	RAFF	SU+HEX	UNK
<i>min</i>			% of total activity			
70	1	7.0	60.7	6.6	23.0	2.7
30	2	4.1	77.1	2.5	16.3	0.0
15	3	10.3	70.1	1.8	17.8	0.0

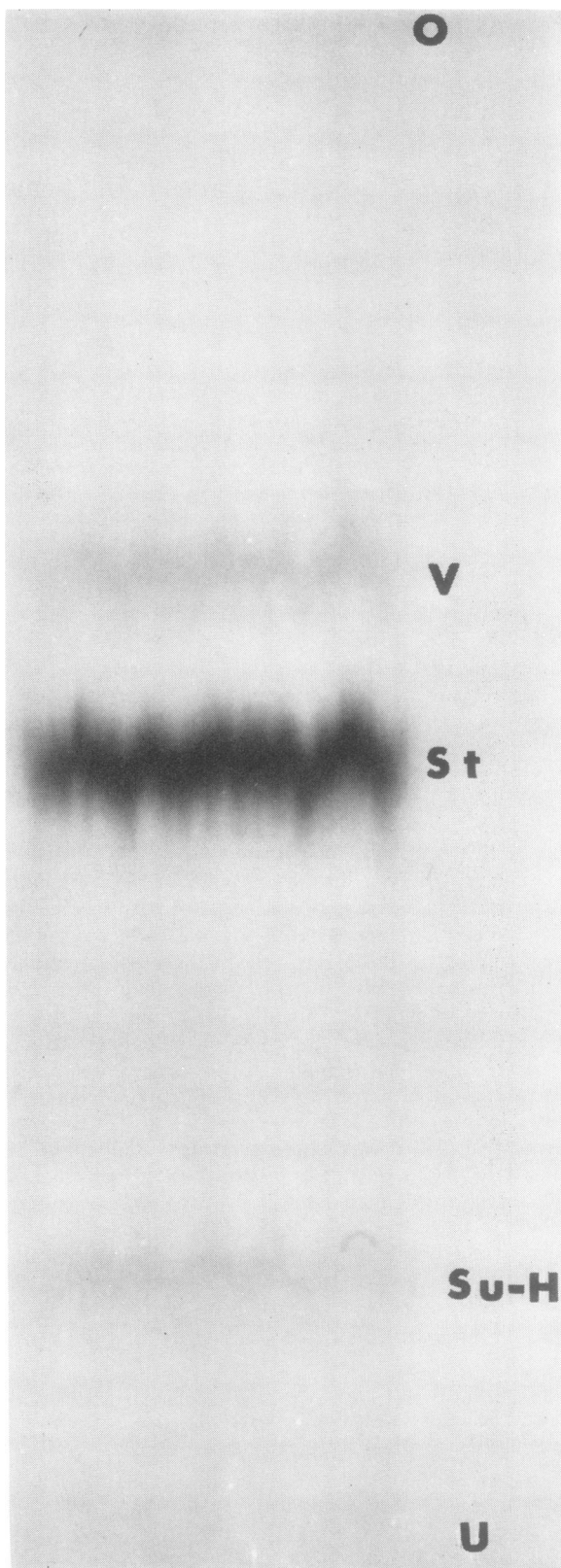


FIG. 1. Autoradiogram of a BAAW 1:1:1 chromatogram of petiole extract. O, origin; V, verbascone; St, stachyose; Su-H, sucrose and hexoses; U, unknown.

integrating the area under each peak and subtracting background. Final location of the labeled substances was obtained by autoradiography (fig 1).

**Hydrolysis and Separation of Products.** The stachyose from adjacent 2 cm sections of petiole was combined; *i.e.*, 1 + 2, 3 + 4, etc. and incubated at  $35 \pm 1^\circ$  for 4 hours in 2 ml of 0.05 M Na-acetate buffered at pH 4.5 with 400  $\mu$ g of "Invertase with Melibiase" from Nutritional Biochemicals Corporation, Cleveland, Ohio. To facilitate chromatography of the hydrolysate, the protein was removed by adding 1 ml of a slurry of Dowex-50 ion exchange resin (1:1 resin:water, v/v)  $H^+$  form to the chilled incubation mixture. After 5 minutes, the mixture was filtered through a Millipore filter. If extreme care had not been taken to remove all  $Cl^-$  from the resin (as tested by  $AgNO_3$ ) severe degradation of hexoses occurred.

The hydrolyzed, protein free samples were chromatographed for 5 days with BEW, scanned and placed on film. The  $R_F$  values in this system, taking sucrose as 1.00 were: galactose, 1.55; glucose, 1.79; and fructose, 2.38. Figure 2 shows that only part of the stachyose  $^{14}C$  was recovered in the hexoses; darkened areas between the origin and galactose are considered to represent products of partial hydrolysis. Labeled material(s) at the origin is believed to have resulted from hexose degradation, for a greater proportion of the  $^{14}C$  remained at the origin if the time in the enzyme solution was extended.

**Chemical and Radioisotope Analysis.** To each eluted and dried hexose sample, 1 ml of water was added; and from this 25  $\mu$ liters were removed for scintillation counting; the remainder was retained for chemical analysis.

Glucostat and Galactostat enzyme systems from Worthington Biochemical Corporation, Freehold, New Jersey, were used for the quantitative analysis of glucose and galactose, respectively. The analysis of glucose was essentially as recommended by the supplier, with incubation at  $35 \pm 1^\circ$  for 1 hour after which the absorbance was read at 400  $m\mu$ . Contrary to the instructions for the Galactostat system, which indicated the enzyme and the chromogen should be added to the incubation mixture at the start of incubation, the chromogen was added 40 minutes after the enzyme. The complete mixture was incubated an additional 20 minutes, then absorbance read at 425  $m\mu$ . If both galactose reagents were added at the start of incubation, the reaction occurred very slowly over a period of several hours, reaching no obvious point of completion.

Fructose analysis was accomplished using "supercalsul" (glucose-6-P dehydrogenase with hexokinase) in combination with phosphoglucose isomerase from Calbiochemical, Los Angeles, California. Incubation was for 40 minutes at  $35 \pm 1^\circ$ ; samples were read at 340  $m\mu$ . Corrections were made for extraneous material which had eluted from the chromatography paper and which absorbed at 340  $m\mu$ .

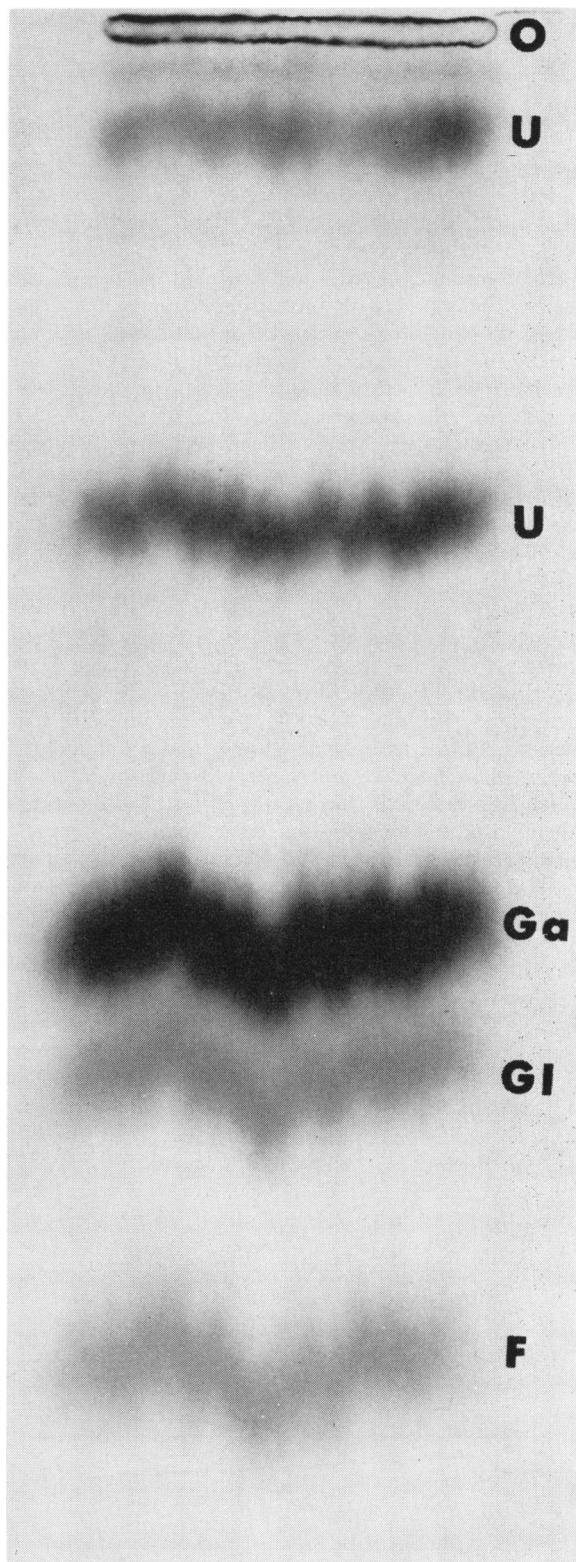


FIG. 2. Autoradiogram of a BEW chromatogram of stachyose hydrolysate. O, origin; U, unidentified, likely partial hydrolysis products; Ga, galactose; Gl, glucose; F, fructose.

## Results and Discussion

*Chemical Distribution of  $^{14}\text{C}$  in the Petioles.* The data in table I were obtained from the scans of the chromatograms of petiole segments. These chromatograms were developed with 1:1:1 BAAW for 24 hours; therefore, separation of sucrose from hexoses did not occur.

The volume of the non-vascular tissue in the petioles increased with increasing distance from the blade, thereby providing more accumulative volume, which, in the 70 minute experiment, accounted for the increase in extractable  $^{14}\text{C}$  with increasing distance from the source (table I). In the shorter experiments, there has not been sufficient time for this intervascular tissue to accumulate significant activity; in fact, the phloem of the petiole had not yet reached equilibrium activity, thereby explaining the decreasing activity with increasing distance from the blade.

Table II presents the distribution of the  $^{14}\text{C}$  among the extracted compounds. Even though these ratios were determined for each section, the data are presented in this form for there were no large differences in the labeling patterns in different sections, except as indicated below. The "unknown" indicated in table II and figure 1 represent at least 2 labeled materials; since these materials became labeled in the petiole after more than 30 minutes, they are considered to be derivatives of transport molecules. In the petiole labeled for 15 minutes there was no detectable  $^{14}\text{C}$  in raffinose more than 16 cm from the blade, and by 20 cm, all the activity was in stachyose (80 %) and verbascose (20 %).

From these data it seems reasonable to assume that stachyose was a major carrier of  $^{14}\text{C}$ , and that verbascose was of secondary importance. Because of the small amounts of label in raffinose, it appears to have been of little importance in carbon transport. If the conclusion from the literature, that hexoses are unimportant in transport, is accepted (8, 15, 25), one must assume that the labeled hexoses were produced from transported molecules. About half of the sucrose-hexose peaks represented labeled sucrose, as determined for a few samples by rechroma-

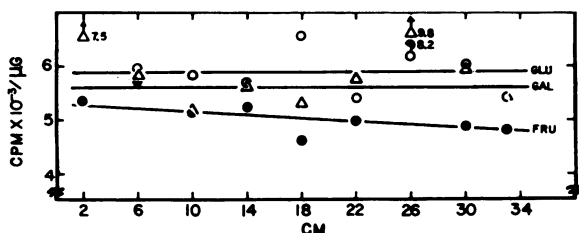


FIG. 3. Specific activities of hexoses derived from stachyose hydrolysis after 70 minutes labeling.  $\Delta$ , Galactose;  $\circ$ , glucose;  $\bullet$ , fructose. CM indicates distance from the source blade to the center of the sample.

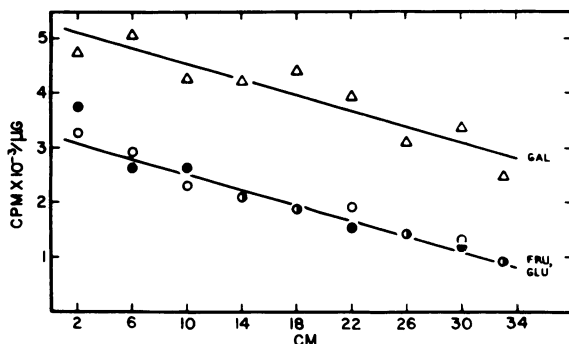


FIG. 4. As figure 3 for 30 minutes labeling. Data from different experiment than data in tables I and II.

tographing the sugars of this peak in BEW. The origin of this sucrose remains unclear.

*Specific Activity of the Hexose Moieties of Stachyose.* The specific activities (cpm/ $\mu\text{g}$ ) of the hexoses derived from stachyose plotted against distance from the source blades are presented in figures 3 through 5. These curves are similar to those presented by Mortimer (11) for total  $^{14}\text{C}$  in beet petioles, in that the amount of  $^{14}\text{C}$  decreased linearly with translocation distance rather than the more usual logarithmic decreases reported in the literature (3, 4, 15). The data presented in figures 4 and 5 are not from the same plants represented for the 30 and 15 minute labeling experiments in tables I and II.

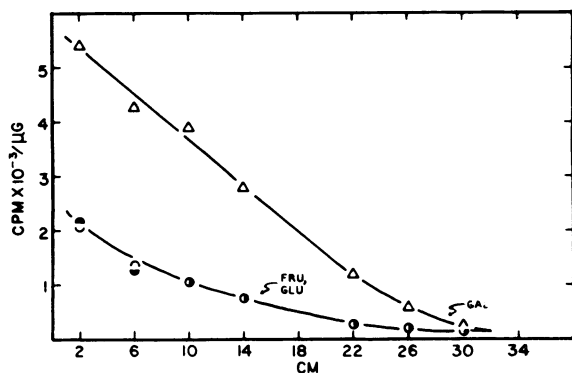


FIG. 5. As figure 3 for 15 minutes labeling. Data from different experiment than data in tables I and II.

After 70 minutes of labeling (fig 3), the specific activities of the 3 hexoses were essentially the same, and were fairly constant throughout the length of the petiole. For reasons discussed below, the indicated slight decrease in fructose specific activity with increasing distance from the blade is not believed to be real.

After 15 minutes of labeling (fig 5), galactose was more highly labeled than were the other 2 hexose moieties of stachyose. Near the front of radioactivity, the curves become non-linear, resulting in a convergence as zero is approached. This would

be expected, for all compounds, into which there is an entry pathway, will become immediately labeled, to some degree, upon exposure to  $^{14}\text{CO}_2$ .

After 30 minutes of labeling (fig 4) the specific activities of all the hexose moieties declined with increasing distance from the source. Although the specific activities of fructose and glucose were lower than that of galactose, the rates of decline were quite similar. Since the stachyose isolated from the petiole was presumably synthesized in the blade, the study of the labeling pattern of stachyose in the petiole is a historical study (in minutes) of the past labeling pattern in the blade. Considering the distance along the petiole axis as a time axis extending into the past (fig 4), and extrapolating these curves to zero activity, galactose activity would extend farther into the past than would the activities of the other 2 hexoses. This indicates that the galactose moiety of stachyose became labeled before the glucose and fructose moieties. This steady state increase in  $^{14}\text{C}$  content of all the hexose moieties of stachyose is important in the development of theories about synthetic pathways. These labeling patterns are in agreement with those reported by Kandler (7) and Senger and Kandler (14), in stachyose extracted from *Catalpa* and *Lamium* leaves.

Within any 1 section of petiole from plants labeled 15 and 30 minutes, the specific activities of the glucose and fructose moieties of stachyose were essentially the same. It would seem unreasonable that these moieties should be equally labeled in the shorter experiments and unequally labeled in the longer experiments; therefore, it is assumed that data for the 70 minute experiment showing a slightly lower specific activity for fructose than for glucose represents a random fluctuation. Kandler's data (9) and data from other experiments not reported here support this conclusion.

Weidner (22) tentatively concluded that stachyose was the only carrier of significant quantities of carbon in cucumber. This conclusion was based on a time-course study of the increase in the  $^{14}\text{C}$  content in the various compounds in young developing leaves into which  $^{14}\text{C}$  was being transported from a mature source blade. Weidner's plots of the percent  $^{14}\text{C}$  in each compound, except stachyose, extrapolated to zero at the time of the initial appearance of  $^{14}\text{C}$ . In similar plots of  $^{14}\text{C}$  in the various hexose moieties of stachyose, the curve for galactose would extrapolate toward 100 % of the total. This does not establish the galactose moiety of stachyose as the only carrier of carbon in the stachyose molecule; however, it does reflect the sequence of labeling of the hexose moieties during stachyose synthesis. If, regardless of their chemical combination, the time sequence of labeling of all hexoses moieties in the blade is the same as that in stachyose, then  $^{14}\text{C}$  would appear in stachyose (in the galactose moiety) before it could appear in sucrose. This labeling sequence would permit sucrose, as well as stachyose to be transported, yet the first detectable label in a

sink would be in stachyose. This only permits, but does not require that sucrose is a significant carbon carrier in cucurbits. It should be noted that Weidner indicated this alternative.

Recently there have been reports of *in-vitro* enzymatic synthesis of raffinose (1,10,12). The most reasonable system for the net synthesis of raffinose was proposed by Pridham and his co-workers (1,12). This involved the transfer of galactose to sucrose from UDP-galactose. It would be attractive to hypothesize that a second galactose unit could be transferred, thereby forming stachyose. These workers recovered no stachyose from their enzyme system, even though the source of the enzymes, immature seeds of *Vicia faba*, produced more stachyose than raffinose (1).

Tanner and Kandler (17) have obtained synthesis of stachyose by an enzyme preparation from ripening seeds of dwarf beans. The donor of the galactose to raffinose was galactinol (*O*- $\alpha$ -D-galactopyranosyl-(1 $\rightarrow$ 1)-myo-inositol). This was formed from UDP-galactose and myo-inositol. Kandler (7) has combined these sequences of raffinose and stachyose synthesis in proposing a pathway for stachyose synthesis which could account for the results reported here.

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### Literature Cited

1. BOURNE, E. J., M. W. WALTER, AND J. B. PRIDHAM. 1965. The biosynthesis of raffinose. *Biochem. J.* 97: 802-06.
2. BURLEY, J. W. A. 1961. Carbohydrate translocation in raspberry and soybean. *Plant Physiol.* 36: 820-24.
3. CANNY, M. J. 1961. Measurements of the velocity of translocation. *Ann. Botany London* 25: 153-67.
4. CANNY, M. J. 1962. The translocation profile: Sucrose and carbon dioxide. *Ann. Botany London* 26: 182-96.
5. GEIGER, D. R. AND C. A. SWANSON. 1965. Evaluation of selected parameters in a sugar beet translocation system. *Plant Physiol.* 40: 942-47.
6. HATCH, M. D. AND K. T. GLASZIOU. 1964. Direct evidence for translocation of sucrose in sugarcane leaves and stems. *Plant Physiol.* 39: 180-84.
7. KANDLER, O. 1967. Biosynthesis of poly- and oligosaccharides during photosynthesis in green plants. *Harvesting The Sun*. A. San Pietro, F. A. Greer, and T. J. Army, eds. Academic Press, New York. 131-52.
8. KURSANOV, A. L. 1963. Metabolism and the transport of organic substances in the phloem. *Advan. Bot. Res.* 1: 209-78.

9. MEYER, B. S., D. B. ANDERSON, AND C. A. SWANSON. 1955. Laboratory Plant Physiology. D. Van Nostrand Company, Incorporated, Princeton, New Jersey.
10. MORENO, A. AND C. E. CARDINI. 1966. A raffinose-sucrose transgalactosidase from wheat germ. *Plant Physiol.* 41: 909-10.
11. MORTIMER, D. C. 1965. Translocation of the products of photosynthesis in sugar beet petioles. *Can. J. Botany* 43: 269-80.
12. PRIDHAM, J. B. AND W. Z. HASSID. 1965. Raffinose biosynthesis. *Plant Physiol.* 40: 984-86.
13. PRISTUPA, N. A. 1957. The transport form of carbohydrates in pumpkin plants. *Fiziol. Rast.* (English transl.) 6: 26-32.
14. SENSER, M. AND O. KANDLER. 1967. Galactinol, ein Galactosyldonor Für die biosynthesis der Zuckenden Raffinosefamilie in Blättern. *Z. Pflanzenphysiol.* 57: 376-88.
15. SWANSON, C. A. 1959. Translocation of Organic Solutes. *Plant Physiology: A Treatise*. Vol. 2. F. C. Steward, ed. Academic Press, New York. 481-551.
16. SWANSON, C. A. AND E. D. H. EL SHISHINY. 1958. Translocation of sugars in the concord grape. *Plant Physiol.* 33: 33-37.
17. TANNER, W. AND O. KANDLER. 1966. Biosynthesis of stachyose in *Phaseolus vulgaris*. *Plant Physiol.* 41: 1540-42.
18. TRIP, P., C. D. NELSON, AND G. KROTKOV. 1965. Selective and preferential translocation of  $^{14}\text{C}$ -labeled sugars in white ash and lilac. *Plant Physiol.* 40: 740-47.
19. WEBB, J. A. AND P. R. GORHAM. 1964. Translocation of photosynthetically assimilated  $^{14}\text{C}$  in straight-necked squash. *Plant Physiol.* 39: 663-72.
20. WEBB, J. A. AND P. R. GORHAM. 1964. Stachyose transport in plants. *Plant Physiol.* 39: 973-77.
21. WEBB, J. A. AND P. R. GORHAM. 1965. Radial movement of  $^{14}\text{C}$ -translocates from squash phloem. *Can. J. Botany* 43: 97-103.
22. WEIDNER, T. M. 1964. Translocation of photosynthetically labeled  $^{14}\text{C}$  compounds in bean, cucumber, and white ash. Ph.D. Thesis. The Ohio State University, Columbus, Ohio.
23. ZIMMERMANN, M. H. 1957. Translocation of organic substances in trees. I. The nature of sugars in the sieve tube exudate of trees. *Plant Physiol.* 32: 288-91.
24. ZIMMERMANN, M. H. 1957. Translocation of organic substances in trees. II. On the translocation mechanism in phloem of white ash (*Fraxinus americana* L.). *Plant Physiol.* 32: 399-404.
25. ZIMMERMANN, M. H. 1960. Transport in the phloem. *Ann. Rev. Plant Physiol.* 11: 167-90.